Comparison of *in vitro* models to study bacterial adhesion to the intestinal epithelium

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ABSTRACT

Aims: To evaluate the adhesion ability of intestinal bacteria to different *in vitro* models of intestinal epithelia, and to estimate the suitability of these models and the type of interactions involved.

Methods and results: The adhesion of probiotic (*L. rhamnosus* GG and *B. animalis* subsp. *lactis* Bb12), commensal (*B. animalis* IATA-A2 and *B. bifidum* IATA-ES2) and potentially pathogenic bacteria (*E. coli* and *L. monocytogenes*) was determined. The adhesion models used were polycarbonate-well plates, with or without mucin, and different configurations of Caco-2 and/or HT29-MTX cell cultures. All bacteria adhered to wells without mucin (2.6-27.3%), the values being highly variable depending on the bacterial strain. Adhesion percentages of potentially probiotic bacteria to Caco-2 cultures were remarkably lower ($P<0.05$) than those to mucin, and more similar to those of pathogenic strains. The lowest adhesion of different bacterial strains was detected on HT29-MTX (0.5-2.3%) cultures and Caco-2/HT29-MTX (0.6-3.2%) cocultures, while these values were increased in Caco-2 cultures plus mucin.

Conclusions: The results suggest that bacterial strains exhibit different capacity to adhere to cellular components and several types of mucin present in different models, showing preference for intestinal MUC2.

Significance and impact of the study: The use of Caco-2 cells monolayer plus mucin (type II) better approaches the physiological characteristics of *in vivo* situation, providing a suitable *in vitro* model to evaluate bacterial adhesion.

Keywords: Probiotics, adhesion, Caco-2, HT29-MTX, intestinal mucin.

INTRODUCTION

There is growing interest in the design of functional foods beneficial to human health. Within the context of functional foods, it is known that probiotic bacteria have desirable traits such as immunomodulatory properties and ability to inhibit pathogenic microorganisms by different mechanisms (Sanz *et al*., 2007). Bacterial adhesion to the intestinal epithelium is considered a requisite for probiotic selection, since it may influence the residence time of the bacteria in the intestinal tract (Servin and Coconnier, 2003). The ability of some probiotic strains to inhibit pathogen colonization and invasion and to modulate the immune response(s) has also been related to their ability to adhere to intestinal mucus and/or epithelial cells (Servin and Coconnier, 2003). In a similar way as beneficial bacteria, the harmful effect of enteropathogen bacteria may be determined by their ability to adhere and colonize the intestinal epithelium.

Although human clinical trials are the definitive tool to establish probiotic functionality, the use of *in vitro* models is necessary to select the most promising strains prior to such trials. Several *in vitro* studies have been made to evaluate the adhesion ability of potential probiotic bacteria and their interactions with pathogens at the intestinal epithelial interface (Sanz *et al*., 2007; Izquierdo *et al*., 2008; Sánchez, *et al*., 2008). The simplest model to evaluate the adherence of bacterial strains to intestinal mucus is based on the immobilization of commercially available mucin on a micro-well plate surface (Tuomola *et al*., 1999; Izquierdo *et al*., 2008). However, cultures of human intestinal epithelial cell lines, notably Caco-2 and HT29-MTX cells derived from colon adenocarcinoma, seem to better represent the *in vivo* situation. More concisely Caco-2 cell cultures has allowed important advances in cellular interaction studies since they grow in culture forming a homogeneous and polarized cell monolayer, which resembles mature human enterocytes in the small intestine (Pinto *et al*., 1983; Lenaerts *et al*., 2007). A previous study proved that fluorescent labelling of bacteria in conjunction with Caco-2 cell monolayers is a suitable model for adhesion studies and provides a reliable and safer alternative to radioactive labelling of bacteria (Bianchi *et al*., 2004). However, the lack of an adequate mucus layer constitutes a major drawback for the use of this cell line. The HT29-MTX cell line resulted from the isolation of HT29 cells adapted to methotrexate (MTX) (Lesuffleur *et al*., 1990), which differentiate into goblet cells characterized by the secretion of mucin (Lesuffleur *et al*., 1993; Leteurtre *et al*., 2004). In the intestinal epithelium, enterocytes and goblet cells represent the two major cell phenotypes. To better approach the cell population representing the intestinal

epithelium, co-cultures of Caco-2 cells and mucin-secreting HT29-MTX cells have also been developed (Walter *et al*., 1996; Pontier *et al*., 2001; Novellaux *et al*., 2006; Mahler *et al*., 2008). Nevertheless, the use of combined Caco-2/HT29-MTX culture systems to evaluate bacterial adhesion of potentially probiotic strains has not been reported.

The objective of this study was to compare the adhesion ability of probiotic, commensal and pathogenic bacterial strains to different *in vitro* models of intestinal epithelium. Polycarbonate-well plates treated or not with mucin and different configurations of Caco-2 and/or HT29-MTX cell cultures, to determine their suitability and the possible type of interactions involved in adhesion were used.

MATERIAL AND METHODS

Bacterial cultures. *Bifidobacterium bifidum* (IATA-ES1), and *Bifidobacterium animalis* (IATA-A2) strains were isolated from faeces of healthy human subjects and identified as described elsewhere (Medina *et al*., 2008). *Bifidobacterium animalis* subsp. *lactis* Bb12 (Chr. Hansen, Horsholm, Denmark) and *Lactobacillus rhamnosus* GG (ATCC 53103) were included as controls because they were reported to show high adhesion ability (Izquierdo *et al*., 2008). Bifidobacteria and lactobacilli were grown in Man-Rogosa-Sharpe broth and agar (Scharlau, Barcelona, Spain) supplemented with 0.05% (w/v) cysteine (Sigma, St. Louis, MO), and incubated at 37ºC under anaerobic conditions (AnaeroGen; Oxoid, Basingstoke, UK). *Escherichia coli* strains, IATA-CBL2 and IATA-CBD10, were also isolated from faeces (Sánchez *et al*., 2008). They were grown in Brain-Heart broth and agar (Scharlau), and incubated at 37ºC under aerobic conditions. *Listeria monocytogenes* CECT 935 was grown in Brain-Heart broth and agar, and incubated at 37ºC under aerobic conditions. All cultures were grown for 20 h to be used in adhesion experiments.

Mucin treatment. Crude mucin (Type II, Sigma-Aldrich) was diluted in a phosphate buffered solution (pH 7.2) (PBS) (0.5 mg/ml). An aliquot (0.5 ml) of this solution was loaded into polycarbonate 24-well plates (Costar, Cambridge, MA, USA), with and without Caco-2 cell cultures, and incubated at 37ºC for 1 h. To remove unbound mucin, the wells were washed twice with 0.5 ml PBS.

Cell lines and culture configurations. The Caco-2 cell line was obtained from the American Type Culture Collection (Rockville, MD, USA) at passage 17 and used in experiments at passage 25-33. The HT29-MTX cell line was kindly provided by Dr. T. Lesuffleur (INSERM U560, Lille, France) at passage 7 and used in experiments at

passage 16-22. Caco-2, HT29-MTX cells and Caco-2 cocultured with HT29-MTX cells were grown in Dulbecco's Modified Eagle Medium (DMEM Glutamax, Gibco, Gibco, Rockville, MD, USA) containing 4.5 g/l glucose, and supplemented with 25 mM HEPES buffer, and 10% (v/v) fetal bovine serum (Gibco). The cells were maintained at 37°C in 5% CO_2, 95% air and the culture media was changed every 2 days.

For adhesion experiments, Caco-2 and HT29-MTX cells were cultured separately, or together at 90:10, Caco-2:HT29-MTX, ratios to mimic the major cell types in the intestine. Cells were seeded at a density of 50,000 cells/cm^2 onto 24-well plates (Costar). All cultures were grown in DMEM, and culture media was changed every two days. Experiments were performed 15 days post seeding, after complete morphological and functional differentiation of Caco-2 cells (Jovaní *et al*., 2001; Mahler *et al*., 2008).

Adhesion assay. Bacteria from 20-hour-old cultures were collected by centrifugation (4,000 x *g* for 5 minutes at 4ºC), washed twice and resuspended in PBS to reach an optical density of 0.5 ($A_{\lambda 600}$). Colony forming unit (CFU/ml) equivalence was determined by plate count, and the size of inoculum ranged between 6.0 x10^7 and 8.2 x10^8 CFU/ml. Suspensions of different bacteria were incubated with 75 µmol l^{-1} carboxyfluorescein diacetate (CFDA) (Sigma-Aldrich), at 37ºC for 30 minutes. CFDA serves as substrate for intracellular esterases releasing the fluorescent compound 2,7-dichlorofluorescein, thereby, evidencing the presence of live bacteria (Bianchi *et al*., 2004). Then, the mixtures were washed twice and resuspended in PBS. Afterwards, a volume of 0.5 ml working labelled suspensions was loaded into 24-well plates and incubated at 37ºC for 1 hour. After the incubation period, the media was removed and wells were washed twice with 0.5 ml PBS. Then, wells were added with 1 ml 1% (w/v) sodium dodecyl sulphate in 0.1 mol l^{-1} NaOH and incubated at 37ºC for 1 hour. Afterwards, the mixtures were homogenized by pipetting and 0.3 ml of the supernatants were transferred to black 96-well plates. The fluorescence was read in a multiscan fluorometer (Fluoroskan Ascent, Labsystem, Oy, Finland) at λ_{ex} 485 and λ_{em} 538 nm. In parallel the bacterial adhesion was also evaluated in 24-well plates without mucin. In order to evaluate the potential CFDA unspecific adsorption to the wells, negative controls without bacteria were used throughout the experiment.

Adhesion was expressed as the percentage of fluorescence recovered after binding to mucin and/or cell cultures relative to the fluorescence of the bacterial suspension added to the wells. Each assay was performed in triplicate, and conducted in two independent experiments.

Statistical analysis. One-way analysis of variance (ANOVA) and the Tukey *post hoc* test were applied. Statistical significance was established at $p<0.05$. SPSS v.15 (SPSS Inc., Chicago, IL, USA) was used for the statistical analysis.

RESULTS

Bacterial adhesion to mucin. The adhesion percentages of different bacterial strains tested, estimated using the classical mucin adhesion assay, are shown in Table 1. All bacteria exhibited adhesion capacity to untreated wells, and *B. lactis* Bb12 showed the highest adhesion percentage. The adhesion percentages of probiotic (*L. rhamnosus* GG and *B. lactis* Bb12) and potentially probiotic (*B. animalis* IATA-A2 and *B. bifidum* IATA-ES2) strains (9.9 to 27.3%) were significantly higher ($P<0.05$) than those of potentially pathogenic strains such as *E. coli* (IATA-CBL2 and -CBD10) and *L. monocytogenes* CECT 935 (2.6-5.9%). The adhesion values of bacterial strains to mucin (type II)-treated wells were similar as those (2.7 to 31.1%) detected in untreated wells. *B. lactis* Bb12 was also the strain showing the highest adhesion capacity to mucin-treated wells. *B. lactis* Bb12 and *B. animalis* IATA-A2 showed higher ($P<0.05$) adhesion percentages to mucin than to untreated wells. In contrast, *B. bifidum* IATA-ES2, *E. coli* IATA-CBL2, and *L. monocytogenes* CECT 935 showed lower ($p<0.05$) adhesion percentages to mucin than to untreated polycarbonate wells.

Bacterial adhesion to cell cultures. The relative adhesion of different bacterial strains to several cultures of human intestinal epithelial cells is shown in Table 1. The adhesion values of different bacterial strains to Caco-2 cell cultures showed less variability than those detected with well-plates treated or not with mucin. Adhesion percentages of *L. rhamnosus* GG, *B. lactis* Bb12, and *B. animalis* IATA-A2 to Caco-2 cultures were remarkably lower ($P<0.05$) than those to mucin, and more similar to those of the *E. coli* strains and *L. monocytogenes*. The adhesion percentages of *B. bifidum* IATA-ES2 and *L. monocytogenes* CECT 935 to Caco-2 and mucin did not differ ($P>0.05$). In contrast, adhesion of both *E. coli* strains tested was higher ($P<0.05$) to Caco-2 cultures than to mucin, indicating that interaction with cellular components could be more relevant than to mucin to *E. coli* adhesion.

The use of HT29-MTX cell cultures, a mucin secreting cell line, markedly reduced the adhesion values of all bacterial strains in relation to those obtained with Caco-2 cell cultures (Table 1). Similarly, low adhesion values to Caco-2:HT29-MTX (90:10) cocultures were detected for all strains without showing statistically significant

differences among them. As shown, the relatively high adhesion percentages of both commercial probiotics *L. rhamnosus* GG and *B. lactis* Bb12 to mucin were also markedly reduced and resulted similar ($p>0.05$) in both HT29-MTX cultures or Caco-2:HT29-MTX (90:10) cocultures. The addition of mucin to Caco-2 cells significantly reduced ($p>0.05$) the adhesion of *L. rhamnosus* GG and two *E. coli* strains, probably due to the ability of this glycoprotein to overlap adhesion sites in Caco-2 cells.

DISCUSSION

Bacterial adhesion to the intestinal epithelium influences the residence time and the ability of probiotic strains to modulate the immune response(s) and, thereby, to exert health effects in the gut (Servin and Coconnier, 2003). The results indicate that interactions with glycoproteins partly explain the adhesion ability of probiotic, commensal and pathogen bacterial strains and that this feature is partially shared within the same species. In addition, adhesion percentages to untreated polycarbonate wells suggests that hydrophobic interactions are relevant to their adhesion ability. In fact, *B. bifidum* IATA-ES2 and *L. monocytogenes* CECT 935, showed adhesion capacities 2.1- and 1.9-fold higher to untreated wells than to mucin-treated wells. The adhesion percentages to mucin of the commercially available probiotic bacteria, *L. rhamnosus* GG (10.2% versus 19.0%) and *B. lactis* Bb12 (31.1% versus 38.5), resembled those reported by using human mucus extracted from faecal samples (He *et al*., 2001). This adherence ability was highly variable among strains in accordance with previous data (Izquierdo *et al*., 2008) and, in particular, the adhesion of *B. animalis* IATA-A2 and *B. bifidum* IATA-ES2 to mucin was significant different ($p<0.05$). It has also been reported that faecal bifidobacteria exhibited a widely variable (0.9-14.6%) adhesion ability to human faecal mucus (He *et al*., 2001), and the reported adhesion values are in good accordance with those detected in this study for the *B. animalis* and *B. bifidum* strains tested.

In the classical mucin adhesion assay it is assumed that the used mucin concentration (0.5 mg/ml) covers the surface area into the well to interact with bacteria (Tuomola *et al*., 1999; Izquierdo *et al*., 2008). However, the results shown that adhesion to the well represents a high proportion of the adhesion detected in mucin treated wells. Therefore, the results obtained by using this model could not represent exclusively those resulting from mucin-bacterial interactions, which are supposed to be one of the main determinants of *in vivo* bacterial adhesion.

The use of human intestinal cell lines in culture for evaluating bacterial adhesion provides useful advantages in cellular interaction studies since they resemble the small intestine forming homogeneous cell monlayers (Pinto *et al*., 1983; Lenaerts *et al*., 2007). In general, probiotic bacterial adhesion percentages to Caco-2 cells monolayers were remarkably lower (P<0.05) than those to mucin, and more similar to commensals and pathogens such as *E. coli* and *L. monocytogenes*, respectively. These results seem to suggest the unspecific adhesion of probiotic bacteria to mucin, although we cannot rule out the implication of mucus-binding elements similar to those identified and characterized in *Lactobacillus* (Perea *et al*., 2007). This observation evidences that mucin and could be critical to determine the residence of commensals instead of pathogenic bacteria. The adhesion values of *B. lactis* Bb12 to this Caco-2 model were relatively higher (8.9%) than those (2%) detected for the same strain and model in previous reports (Fernandez *et al*., 2008), which can be explained by differences in methodology regarding the surface area accessible for bacterial adhesion (24- versus 96-well plates). Interestingly, it has been reported that the proportion of adherent relative to the added bacteria over Caco-2 cultures would likely remain unaltered when using concentrations of inocula between 10^6-10^7 CFU ml^{-1} (Bianchi *et al*., 2004). The use of HT29-MTX cell cultures provides some advantages to study the adhesion ability of bacterial strains to the intestinal epithelium. They constitute a mucin secreting cell culture, and express similar protein patterns as Caco-2 cells and the human intestinal epithelium (Lenaerts *et al*., 2007). However, the adhesion ability of the different bacterial strains to independent cultures of the HT29-MTX cells was markedly lower (P<0.05) than that to Caco-2 cell cultures (Table 1). These results suggest that the mucin glycoproteins expressed by this cell type are not the only major determinants of adhesion but probably other proteins in the cell plasmatic membrane of mature enterocytes.

Fully differentiated Caco-2 cells exhibit many of the characteristics of mature enterocytes (Pinto *et al*., 1983), while HT29-MTX cells differentiate into goblet cells (Lesuffleur *et al*., 1990). To better simulate the major presence of columnar absorptive cells relative to the goblet cells in the intestinal epithelium, co-cultures of the two human intestinal cell lines (Caco-2 and HT29-MTX) have been tested to produce a more physiological model, mimicking the major cell phenotypes present in the intestinal epithelium (Pontier *et al*., 2001; Novellaux *et al*., 2006). Mahler *et al*. (2008) have reported that a firmly adherent mucus layer is formed over both HT29-MTX cultures

and Caco-2:HT29-MTX (90:10) cocultures. The fact that the bacterial adhesion values to Caco-2:HT29-MTX (90:10) cocultures were markedly lower than those to Caco-2 cultures indicate again that mucin layer produced by HT29-MTX cells could cover potential recognition components in the plasmatic membrane of Caco-2 cells making them inaccessible for bacterial adhesion.

The differences in bacterial ability to adhere to mucin, HT29-MTX cultures, and Caco-2:HT29-MTX (90:10) cocultures could be due to the different mucin type present in these models. Differentiated goblet cells secrete several types of mucins including MUC5AC and MUC5B, which are the main expressed mucins, and MUC2, which is less abundant (Lesuffleur *et al*., 1993; Leteurtre *et al*., 2004). In contrast, the mucin used for adhesion assays was purely type II. While MUC2 is predominantly expressed in the large intestine and reduced in the upper intestinal tract, MUC5AC and MUC6 are highly expressed in the stomach and upper intestinal tract. The increased adhesion detected in the presence of mucin, rich in MUC2, in comparison with cell cultures expressing other mucin types is in agreement with other studies showing that lactobacilli adhesion to extracted ileal mucus was higher than to duodenal and jejunal mucus, less rich in MUC2 (Li *et al*., 2008). In fact, the addition of mucin (type II) to Caco-2 cell cultures increased the bacterial adhesion values ($p<0.05$) with respect to those obtained with HT29-MTX and Caco-2:HT29-MTX cocultures. However, the addition of mucin to Caco-2 cell cultures could also mask adhesion sites present in Caco-2 cells. In general, the adherence of bacteria to mucin-treated Caco-2 cultures was slightly lower compared to that detected using the untreated Caco-2 cultures except for *B. bifidum* IATA-ES2 ($P<0.05$), which suggests that this strain might bind more preferentially to MUC2 than to other Caco-2 cellular components.

Overall, the differences detected in adhesion depending on the strain and the type of *in vitro* model system used for the evaluation could be due to both differences in the presence of molecules involved in the interaction as well as on the strain aggregation patterns. The nature of the bacteria cell-associated components involved in their adherence to intestinal epithelial cells has not been completely elucidated and different components seemed to be involved. Most studies have indicated that in strains of the genera *Lactobacillus* (Perea *et al*., 2008) and *Bifidobacterium* components of protein nature are the major responsible for bacterial adhesion to the intestinal mucin types and/or epithelial cells (Izquierdo *et al*., 2008). Several exported proteins such as the mucus binding protein Mub of *Lactobacillus reuteri*, the lectin-like mannose adhesin

Msa of *Lactobacillus plantarum* and a sortase-dependent and other surface layer proteins, have been identified as mediators of adhesion. In addition, the involvement of some other compounds such as carbohydrates (Greene and Klaenhammer, 1994) and/or lipoteichoic acids (Granato *et al*., 1999) has also been suggested, which expression and composition may be strain and species dependent. In addition, differences on aggregation patterns of bacterial strains might contribute to explain the differences on their adhesion abilities. For example, lactobacilli seem to adhere forming pairs and clusters, favouring this process (Coconnier *et al*., 1992; Gopal *et al*., 2001; Li *et al*., 2008), and a similar effect has been reported for several *B. longum* strains (Del Re *et al*., 2000).

Although the models used for in vitro adhesion assays present structures probably involved *in vivo* adhesion and, for instance, Caco-2 cell cultures express biologically significant proteins similarly to those expressed in small intestinal scrapings (Lenaerts *et al*., 2007), *in vivo* studies are required to confirm *in vitro* results. Some comparative evaluations of *in vitro* and *in vivo* adhesion ability of probiotic strains have been published (Crociani *et al*., 1995). While in some cases the adhesion experiments of *B. longum* strains (BB536 and ATCC 15707) to Caco-2 cells were in agreement with the *in vivo* intestinal colonization, particularly for *B. longum* BB536 ingested in the form of a fermented milks (Crociani *et al*., 1995), the adhesion test of *B. animalis* to Caco-2 cultures underestimated the bacterial adhesion determined *in vivo*. These differences between the *in vitro* and *in vivo* situation could be due to the lack of suitability of a unique model system to predict adhesion ability of every strain.

In summary, interactions with mucin (MUC2) seemed to be more relevant to the adhesion of potentially probiotic strains than to pathogens. Although, the use of cell cultures instead of extracted mucin better mimic the *in vivo* situation, the selection of the cell type and culture configuration also seems to be important since it determines the nature of adhesion sites in the system. Therefore, the use of different methods to study adhesion *in vitro* could provide more complete information on different bacterial adhesion ability and could help to elucidate the type of interactions and molecules that mediate the host-microbe interactions.

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Table 1. Adhesion of bacterial strains to mucin (type II), Caco-2 cell cultures, HT29-MTX cell cultures and Caco-2:HT29-MTX cell cocultures (seeded in 90:10 ratio). Results are expressed as mean ± standard deviation (n=5). [a-e] Different superscript letter indicates statistically significant ($p<0.05$) differences in a row. [v-z] Different superscript letter indicates statistically significant ($p<0.05$) differences in a column.

	Relative bacterial adhesion to *in vitro* model (%)					
Bacteria	**Polycarbonate[3]**	**Mucin[4]**	**Caco-2[5]**	**HT29-MTX[5]**	**Caco-2/HT29-MTX[6]**	**Caco-2/Mucin[7]**
L. rhamnosus GG	9.89 ± 0.17 [a,v]	10.21 ± 0.36 [a,v]	5.17 ± 1.15 [c,vw]	0.84 ± 0.09 [b,v]	0.85 ± 0.07 [b,v]	3.19 ± 0.59 [d,v]
B. lactis Bb12	27.25 ± 0.45 [a,w]	31.07 ± 1.02 [b,w]	8.89 ± 0.49 [d,x]	0.76 ± 0.06 [c,v]	0.92 ± 0.08 [c,v]	7.96 ± 0.29 [d,w]
B. animalis	10.92 ± 1.56 [a,v]	17.62 ± 0.99 [b,x]	6.21 ± 1.01 [d,w]	0.54 ± 0.05 [c,v]	0.72 ± 0.06 [c,v]	4.53 ± 0.23 [d,xy]
B. bifidum	11.11 ± 0.71 [a,v]	5.28 ± 0.73 [b,y]	5.91 ± 0.11 [b,vw]	2.33 ± 0.19 [c,w]	3.15 ± 0.30 [c,w]	9.82 ± 0.20 [d,z]
E. coli[1]	5.97 ± 0.25 [a,x]	5.06 ± 0.31 [b,y]	6.45 ± 0.51 [a,w]	1.40 ± 0.09 [c,x]	1.34 ± 0.10 [c,x]	5.07 ± 0.02 [b,y]
E. coli[2]	2.60 ± 0.16 [ab,y]	2.70 ± 0.3 [b,z]	5.43 ± 0.25 [d,vw]	1.88 ± 0.11 [ac,w]	1.89 ± 0.29 [c,y]	3.79 ± 0.47 [e,vw]
L. monocytog.	6.56 ± 0.93 [a,x]	3.51 ± 0.14 [b,z]	4.09 ± 0.37 [b,v]	0.68 ± 0.01 [c,v]	0.59 ± 0.02 [cv]	4.07 ± 0.34 [b,vwx]

[1]*IATA-CBL2*; [2]IATA-CBD10; [3]Untreated 24-well plates; [4]Mucin (type II)-treated 24-well plates; [5]Differentiated (15-days post seeding); [6]Differentiated (15-days post seeding) cocultures in a 90:10 ratio to mimic the major cell phenotypes encountered in the intestine; [7]Differentiated (15-days post seeding) Caco-2 cultures plus mucin (type II).